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HIV-1 NL4-3, but Not IIIB, Inhibits JAK3/STAT5 Activation in CD4$^+$ T Cells[1]

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HIV-1 infection leads to T cell dysfunction and apoptosis *in vivo* and *in vitro.* The shared common γ chain of IL-2R and its associated Janus kinase, JAK3, are indispensable for normal T cell function and survival. We have reported that CD4 ligation with HIV gp120 inhibits T cell receptor-induced activation and expression of JAK3. We have also shown that while some strains of HIV-1, such as NL4-3, induce apoptosis of infected CD4$^+$ T cells, other strains, such as HIV-1 IIIB, do not. Interestingly, we show here that infection of CD4$^+$ T cells with HIV-1 NL4-3, but not IIIB, inhibited activation and expression of JAK3. NL4-3-infected T cells were unable to upregulate JAK3 expression following stimulation through TCR/CD3. In addition, NL4-3, but not IIIB, inhibited tyrosine phosphorylation and expression of STAT5, a downstream target of JAK3. These data suggest a correlation between apoptosis of HIV-1-infected T cells and inhibition of the JAK3/STAT5 activation pathway.

Key Words: AIDS/HIV; NL4-3; IIIB; T lymphocyte; signal transduction; JAK3; STAT5.

INTRODUCTION

T cell dysfunction and apoptosis in HIV disease are thought to be the result of direct viral cytopathicity in infected cells and of indirect effects of viral particles and/or gene products on bystander cells (Finkel *et al.,* 1995; Gandhi *et al.,* 1998; Herbein *et al.,* 1998). Ligation of the CD4 receptor with HIV gp120 or anti-CD4 mAb inhibits T cell receptor (TCR)/CD3-induced activation and induces apoptosis in resting T cells (Banda *et al.,* 1992; Liegler and Stites, 1994). The biochemical mechanisms of HIV-1-induced T cell dysfunction and apoptosis are not understood. Recently, we have shown that increases in JAK3 expression and activation induced by antigen receptor ligation are inhibited by prior CD4 ligation by HIV gp120 or anti-CD4 mAb (Selliah and Finkel, 1998). These data suggest that, at least in this *in vitro* system, binding of CD4 by HIV gp120 downregulates activation-induced transcriptional regulation and activation of JAK3 and its target signal transducer and activator of transcription (STATs). *In vivo* evidence for inhibition of the JAK/STAT pathway in HIV disease comes from data of Pericle *et al.* (1998). The authors observed a selective reduction of STAT5B expression in HIV-infected PBMC and reduced expression of STAT1α, STAT5A, and STAT5B in T cells from HIV-seropositive individuals. Collectively, these data argue that T cell dysfunction and apoptosis in HIV disease may be due, in part, to aberrant regulation of the JAK3/STAT5 signaling pathway.

We and others have reported that HIV-1 induces apoptosis of infected CD4$^+$ T cells (Nardelli *et al.,* 1995; Rapaport *et al.,* 1998). Specifically, we have shown that T cells infected with HIV-1 NL4-3 undergo apoptosis, leading to the elimination of productively infected cells in the culture (Rapaport *et al.,* 1998). In contrast, levels of apoptosis in infected cells from IIIB-infected cultures are significantly less than from NL4-3-infected cultures, leading to persistence of *in vitro* infection. Surprisingly, the frequency of apoptotic IIIB-infected cells is even less than that seen in uninfected cultures (Rapaport *et al.,* 1998). The biochemical mechanisms involved in inhibition of apoptosis by HIV-1 IIIB are not understood.

The JAK3/STAT5 signaling pathway has been shown to play a major role in the proliferation and survival of T cells. Studies of genetically deficient mice showed that JAK3 is absolutely essential for common γ-chain (γ_c)-dependent T cell development and γ_c-dependent prevention of thymocyte apoptosis (Sohn *et al.,* 1998; Suzuki *et al.,* 2000; Thomis *et al.,* 1997). Peripheral T cells from JAK3-deficient mice have increased susceptibility to apoptosis and do not proliferate after *in vitro* stimulation with antigens or mitogens (Sohn *et al.,* 1998; Thomis and Berg, 1997; Thomis *et al.,* 1997). Studies of STAT5-deficient mice show that STAT5 is specifically required for cell cycle progression in response to TCR ligation and

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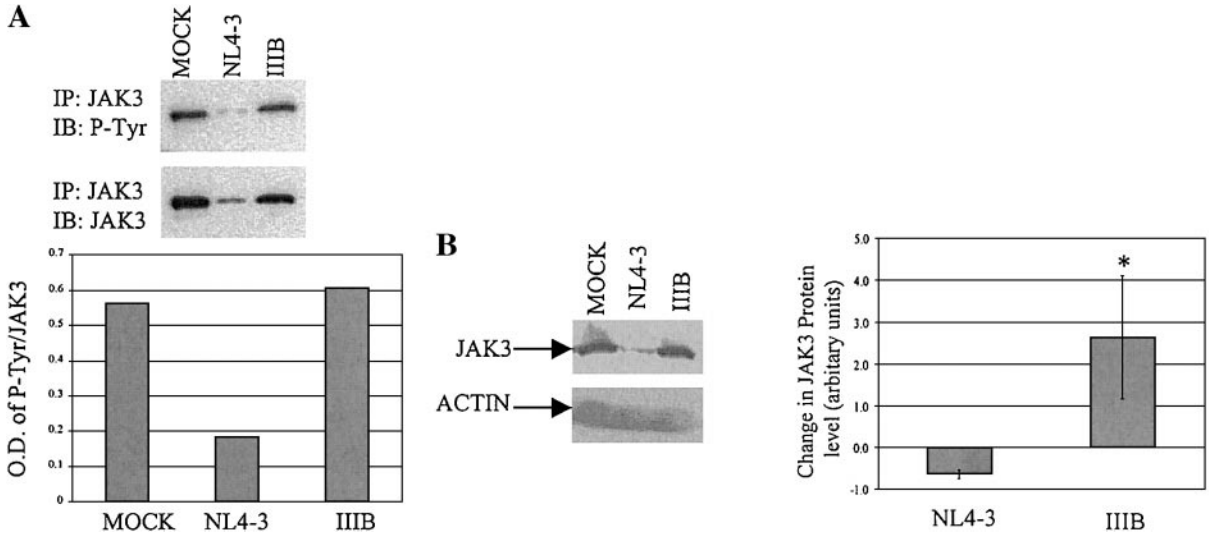

FIG. 1. NL4-3, but not IIIB, inhibits JAK3 activation and expression. (A) JAK3 activation. $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB, and at day 6, cells were harvested and lysed and postnuclear supernatants were immunoprecipitated (IP) with anti-JAK3 pAb. Nitrocellulose membrane was immunoblotted (IB) with anti-phosphotyrosine antibody (P-Tyr, top) and then stripped and immunoblotted with anti-JAK3 antibody (bottom). The OD of each band was determined and the ratio of P-Tyr/JAK3 was plotted. $p24^{gag+}$ cells in NL4-3- and IIIB-infected cultures were 45 and 35%, respectively. A representative experiment of five performed is shown. (B) JAK3 expression. $CD4^+$ T cells were infected with NL4-3 or IIIB as in A. Cell lysates were analyzed by immunoblotting with anti-JAK3 pAb (top) and then stripped and immunoblotted with anti-actin mAb (bottom). The OD of each band was determined and change (increase or decrease) in JAK3 protein level in HIV-infected T cells was compared to the level in uninfected T cells and calculated as described under Materials and Methods. The blot shown is a representative experiment of five and the graph is the average ± SEM of five experiments. The asterisk represents a significant difference between NL4-3 and IIIB ($P = 0.05$).

IL-2 receptor activation (Moriggl *et al.,* 1999). STAT5 is a critical component of the IL-2 receptor-mediated proliferative signal and regulates expression of both mitogenic and survival genes (Lord *et al.,* 2000; Zamorano *et al.,* 1998). Since HIV gp120-induced T cell dysfunction and apoptosis are correlated with inhibition of JAK3 expression and activation, we investigated the effect of NL4-3 and IIIB infection on the JAK3/STAT5 activation pathway.

RESULTS

NL4-3, but not IIIB, inhibits activation and expression of JAK3

CD4 ligation with HIV gp120 inhibits T cell receptor-induced activation and expression of JAK3 (Selliah and Finkel, 1998). Here, we investigated the effect of HIV-1 NL4-3 and IIIB infection of JAK3 activation and expression in $CD4^+$ T cells. Phytohemagglutinin (PHA)-activated $CD4^+$ T cells were infected with NL4-3 or IIIB, and JAK3 activation was determined as described under Materials and Methods. Briefly, postnuclear lysates were immunoprecipitated with anti-JAK3 antibody and immunoblotted with anti-phosphotyrosine antibody. JAK3 is activated following cytokine binding to the cytokine receptor by autophosphorylation on a tyrosine residue (Johnston *et al.,* 1994). NL4-3 infection of T cells inhibited the activation of JAK3 (Fig. 1A). Interestingly, another HIV-1 T cell-tropic virus strain, IIIB, did not inhibit the activation of JAK3 (Fig. 1A). The optical density ratios of tyrosine-phosphorylated JAK3 to total JAK3 protein confirm that NL4-3 infection markedly inhibits JAK3 activation. Numbers of productively infected cells in NL4-3 and IIIB cultures were similar (45 and 35% $p24^{gag+}$ cells, respectively).

Similar experiments were done to determine the expression of JAK3 in NL4-3- or IIIB-infected T cells. JAK3 expression was determined by separating postnuclear lysates on a SDS–PAGE gel and immunoblotting with anti-JAK3 antibody and then with anti-actin mAb. The optical density of each band was measured and the ratio of JAK3/actin was graphed. NL4-3 infection significantly inhibited JAK3 expression in T cells (Fig. 1B). Interestingly, JAK3 levels in IIIB infected cells were similar to the levels in uninfected cells (Fig. 1B). These data show that NL4-3, but not IIIB, inhibits activation and expression of JAK3, one of the kinases necessary for T cell proliferation and survival.

NL4-3, but not IIIB, inhibits TCR/CD3-induced JAK3 expression

Resting T cells have very low levels of JAK3 protein, which increases with activation through the TCR/CD3 complex by an IL-2 receptor-independent mechanism (Johnston *et al.,* 1994). As shown above, PHA-activated T cells have high levels of JAK3 protein (Mock, uninfected

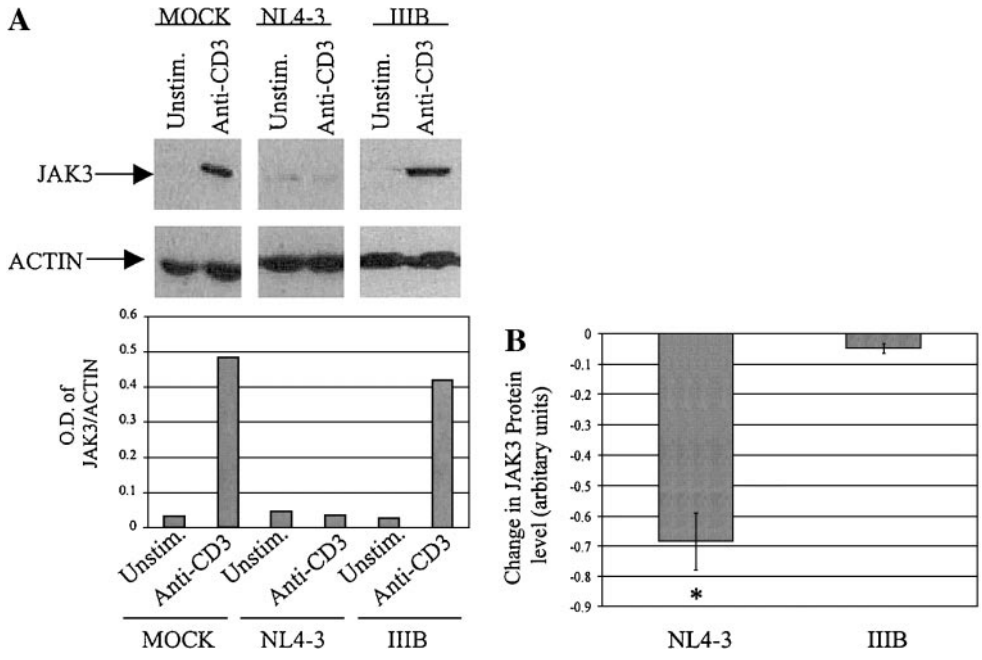

FIG. 2. NL4-3, but not IIIB, inhibits TCR/CD3-induced JAK3 expression. (A) $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB for 4 days and cells were either unstimulated (Unstim.) or stimulated with anti-CD3 mAb for 24 h. Cells were harvested and lysed and postnuclear supernatants were immunoblotted with anti-JAK3 pAb (top) and then with anti-actin mAb (bottom). The OD of each band was determined and the ratio of JAK3/actin was plotted. The percentages $p24^{gag+}$ cells were NL4-3, unstimulated, 20.6; anti-CD3 mAb, 46.4; and IIIB, unstimulated, 13.9; anti-CD3 mAb, 46.8. A representative experiment of three performed is shown. (B) Change in JAK3 protein level in response to anti-CD3 mAb was determined for HIV-infected and uninfected T cells, calculated for each experiment, as described under Materials and Methods. Average ± SEM of three experiments is shown. The asterisk represents a significant difference between NL4-3 and IIIB ($P = 0.0005$).

control T cells), although NL4-3 infection of these cells led to a decrease in JAK3 expression (Fig. 1B). Experiments were done to determine whether these NL4-3-infected T cells could increase JAK3 expression following stimulation through the TCR/CD3 with anti-CD3 mAb. Four days after infection with NL4-3 or IIIB, T cells were either unstimulated or stimulated with anti-CD3 mAb for 24 h. The cells were then harvested, lysed, and analyzed by SDS–PAGE. The nitrocellulose membrane was blotted with anti-JAK3 pAb and then with anti-actin mAb. In T cells cultured without stimulation for 24 h, the JAK3 protein decreased to undetectable levels. In contrast, anti-CD3 mAb stimulation of uninfected T cells significantly increased JAK3 expression (Fig. 2A). Anti-CD3 mAb also induced JAK3 expression in IIIB-infected T cells, to levels similar to those seen in uninfected cells. Interestingly, NL4-3-infected T cells showed no increase in JAK3 expression following anti-CD3 mAb stimulation (Figs. 2A and 2B). These data suggest that NL4-3-infected T cells are defective in activation-induced JAK3 expression.

Inhibition of JAK3 expression correlates with apoptosis in NL4-3-infected T cells

JAK3 has been shown to play a major role in the T cell proliferation and survival of T cells (Kirken *et al.,* 1995). As discussed above, NL4-3, but not IIIB, inhibits JAK3 expression and activation. In order to determine the correlation between JAK3 expression and apoptosis in HIV-infected T cells, the change (increase or decrease) in JAK3 protein level in HIV-infected T cells was compared to the level in uninfected T cells and calculated for each experiment as described under Materials and Methods. The percentage apoptotic cells and percentage $p24^+$ cells were also compared. Significant inhibition of JAK3 expression and increases in apoptosis were seen after day 6 of NL4-3 infection and were significantly different between NL4-3 and IIIB (Fig. 3). In IIIB-infected T cells, no inhibition of JAK3 expression and very low levels of apoptosis were seen. Intriguingly, the JAK3 protein level was higher in IIIB-infected cells than in uninfected cells. We have reported previously that IIIB-infected T cells show less apoptosis than uninfected control T cells (Rapaport *et al.,* 1998). These data show a correlation between JAK3 expression and apoptosis. Further experiments are in progress to directly demonstrate the effect of JAK3 in HIV-induced and spontaneous apoptosis.

NL4-3 inhibits activation, but not expression, of JAK1; NL4-3 does not affect expression or activation of JAK2

JAK1, like JAK3, associates with the IL-2 receptor complex (Russell *et al.,* 1994). Experiments were done to

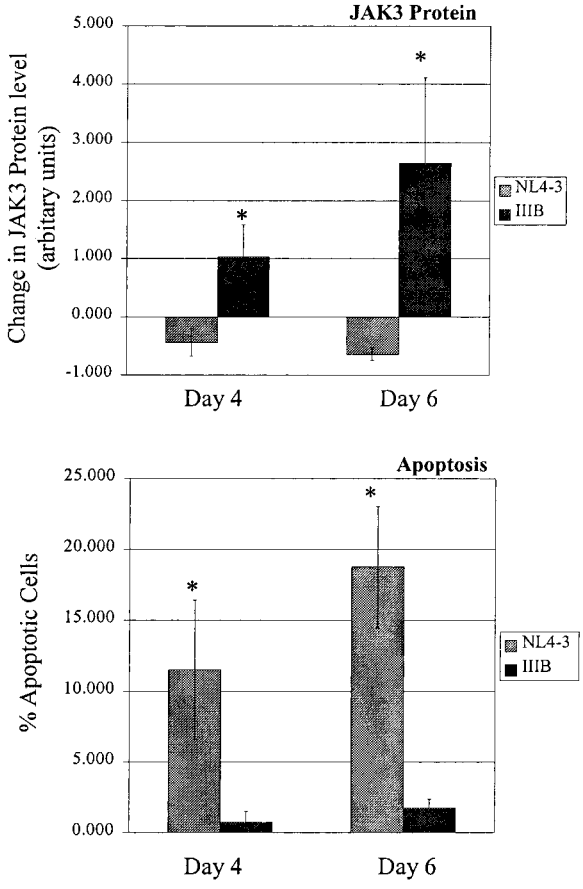

FIG. 3. Inhibition of JAK3 expression correlates with apoptosis in NL4-3-infected T cells. Change (increase or decrease) in JAK3 protein level in HIV-infected T cells (day 4 and day 6 postinfection) was compared to the level in uninfected T cells and calculated for each experiment as described under Materials and Methods (top). Average ± SEM of four experiments is shown. Percentage apoptotic cells was determined with FS/SS analysis for each experiment and average ± SEM of four experiments is shown (bottom). The percentages $p24^{gag+}$ cells, averages ± SEM of four experiments, were day 4, NL4-3, 40.25 ± 5.13; IIIB, 17.73 ± 5.57; and day 6, NL4-3, 25.00 ± 8.63; IIIB = 15.85 ± 4.22. The asterisks represent significant differences between NL4-3 and IIIB ($P < 0.05$).

determine whether NL4-3 inhibits activation and expression of other Janus family kinases, JAK1 and JAK2. PHA-activated $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB and activation and expression of JAK1 and JAK2 were determined as described under Materials and Methods. As shown in Fig. 4A, moderate inhibition of JAK1 activation was seen in NL4-3- but not IIIB-infected T cells. This is intriguing, given recent data suggesting that JAK3 phosphorylates JAK1 (Zhou *et al.,* 2000). In contrast to JAK3, NL4-3 infection did not inhibit the expression of JAK1 (Fig. 4C). No inhibition of JAK2 activation or expression was seen in NL4-3- or IIIB-infected T cells (Figs. 4B and 4C). These data suggest that NL4-3 does not induce a global inhibition of the JAKs, but may specifically target the JAK3 activation pathway.

NL4-3, but not IIIB, inhibits phosphorylation and expression of STAT5

STAT5 is one of the substrates for JAK3, and tyrosine phosphorylation of STAT5 is required for its function as a transcription factor (Darnell, 1997). As with JAK3, STAT5 is required for T cell proliferation and survival (Lord *et al.,* 2000; Moriggl *et al.,* 1999; Zamorano *et al.,* 1998). Since infection of T cells with NL4-3, but not IIIB, inhibited JAK3 activation, we investigated the phosphorylation status of STAT5 in these cells. PHA-activated $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB, and STAT5 phosphorylation was determined as described under Materials and Methods. Briefly, postnuclear lysates were immunoprecipitated with anti-STAT5 antibody and immunoblotted with anti-phosphotyrosine antibody. NL4-3 infection inhibited the tyrosine phosphorylation of STAT5 (Fig. 5A). As expected, IIIB did not inhibit the tyrosine phosphorylation of STAT5. The optical density ratio of tyrosine-phosphorylated STAT5 to total STAT5 protein confirms that NL4-3 markedly inhibits STAT5 phosphorylation. Numbers of productively infected cells in NL4-3 and IIIB cultures were similar (45 and 35% $p24^{gag+}$ cells, respectively).

Similar experiments were done to determine the expression of STAT5 in NL4-3- or IIIB-infected T cells. STAT5 expression was determined by separating postnuclear lysates by SDS–PAGE and immunoblotting with anti-STAT5 pAb and then with anti-actin mAb. The optical density of each band was measured and the ratio of STAT5/actin was graphed. NL4-3, but not IIIB, inhibited STAT5 expression in T cells (Fig. 5B). Anti-STAT5 pAb recognizes both STAT5a and STAT5b isoforms; as shown in Fig. 5B, neither was detected in NL4-3-infected T cells. These data provide further evidence that NL4-3, but not IIIB, inhibits the JAK3/STAT5 activation pathway.

Although in a few experiments, JAK3 and STAT5 proteins were completely absent in NL4-3-infected cells, in a majority of experiments, small amounts of JAK3 and STAT5 protein could be detected. We asked whether this small amount of JAK3 could be activated to phosphorylate its substrate, STAT5, by the addition of a higher concentration of IL-2. This experiment was done to increase the sensitivity of the assay to detect phosphorylated STAT5, thereby indirectly determining the activation status of JAK3. T cells from uninfected or NL4-3- or IIIB-infected cultures were incubated without or with 50 U/ml IL-2 for 15 min at 37°C. Whole-cell lysates were separated by SDS–PAGE and immunoblotted with the indicated antibodies. Addition of IL-2 significantly increased the phosphorylation of STAT5 in uninfected or IIIB-infected T cells (Fig. 6A). In contrast, T cells from NL4-3 infection did not show any detectable phosphorylated STAT5 (Fig. 6). Longer exposure of the blot did not reveal any phosphorylated STAT5 in NL4-3-infected T cells (Fig. 6B, pSTAT5—longer exposure). Measurement

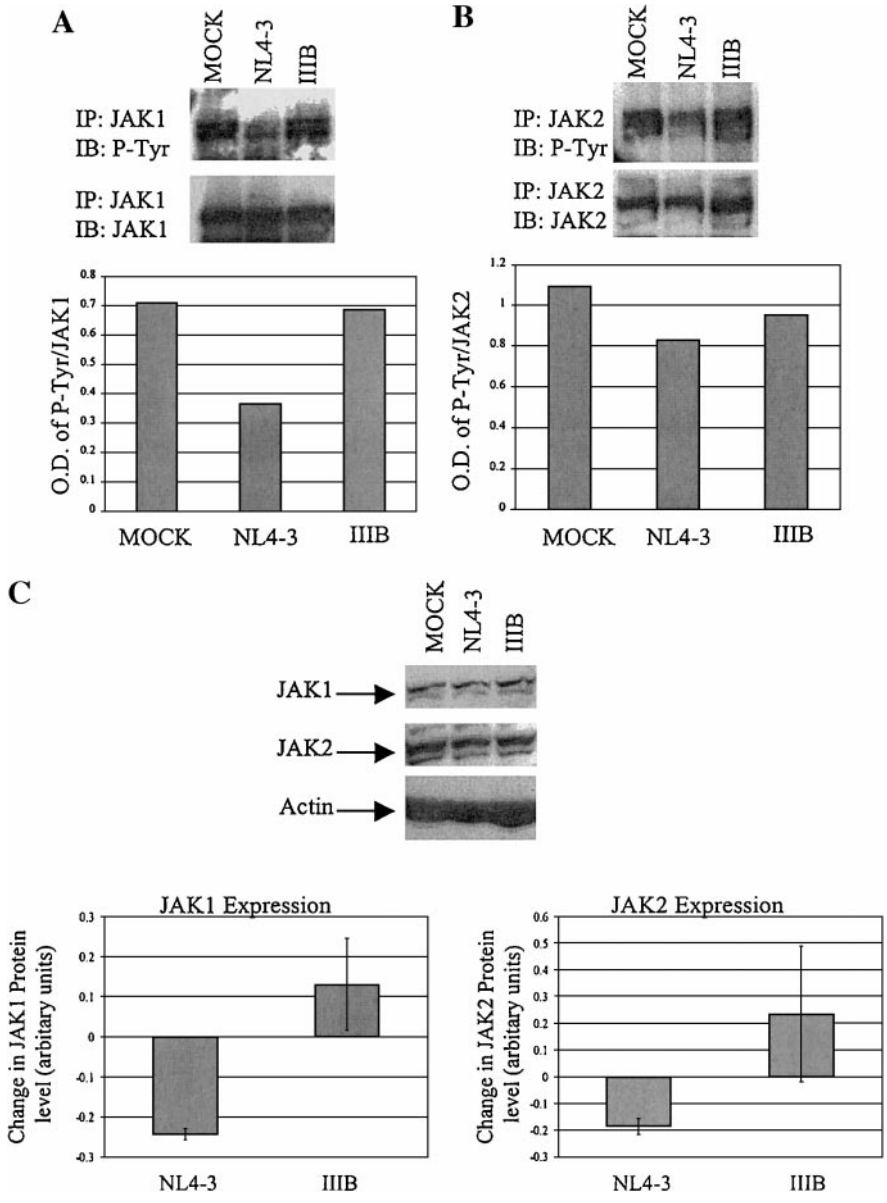

FIG. 4. NL4-3 inhibits activation, but not expression, of JAK1; NL4-3 does not affect expression or activation of JAK2. (A) JAK1 activation. $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB, and at day 6, cells were harvested and lysed and postnuclear supernatants were immunoprecipitated (IP) with anti-JAK1 pAb. Nitrocellulose membrane was immunoblotted (IB) with anti-phosphotyrosine antibody (P-Tyr, top) and then stripped and immunoblotted with anti-JAK1 antibody (bottom). Percentages $p24^{gag+}$ cells in NL4-3- and IIIB-infected cultures were 20.19 and 18.13, respectively. The OD of each band was determined and the ratio of P-Tyr/JAK1 was plotted. A representative experiment of three performed is shown. (B) JAK2 activation. $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB, and at day 6, cells were harvested and lysed and postnuclear supernatants were immunoprecipitated (IP) with anti-JAK2 pAb. Nitrocellulose membrane was immunoblotted (IB) with anti-phosphotyrosine antibody (P-Tyr, top) and then stripped and immunoblotted with anti-JAK2 antibody (bottom). Percentages $p24^{gag+}$ cells in NL4-3- and IIIB-infected cultures were 25.67 and 40.75, respectively. The OD of each band was determined and the ratio of P-Tyr/JAK2 was plotted. A representative experiment of three performed is shown. (C) JAK1 and JAK2 expression. $CD4^+$ T cells were infected with NL4-3 or IIIB as in A. Cell lysates were analyzed by immunoblotting with anti-JAK1 pAb (top), anti-JAK2 pAb (middle), and then anti-actin mAb (bottom). The OD of each band was determined and change in JAK1 or JAK2 protein level in HIV-infected T cells was compared to the level in uninfected T cells and calculated as described under Materials and Methods. The blot shown is a representative experiment of three and the graphs are the averages ± SEM of three experiments. There is no statistically significant difference between NL4-3 and IIIB (for JAK1 expression $P = 0.184$ and for JAK2 expression $P = 0.178$).

of the ratio of pSTAT5/STAT5 OD showed that phosphorylation of STAT5 is completely inhibited in NL4-3-infected T cells (Fig. 6B). These data support our hypothesis that NL4-3 infection of T cells inhibits the JAK3/STAT5 activation pathway by inhibiting JAK3 kinase activity and inhibiting the expression of JAK3 and STAT5 protein.

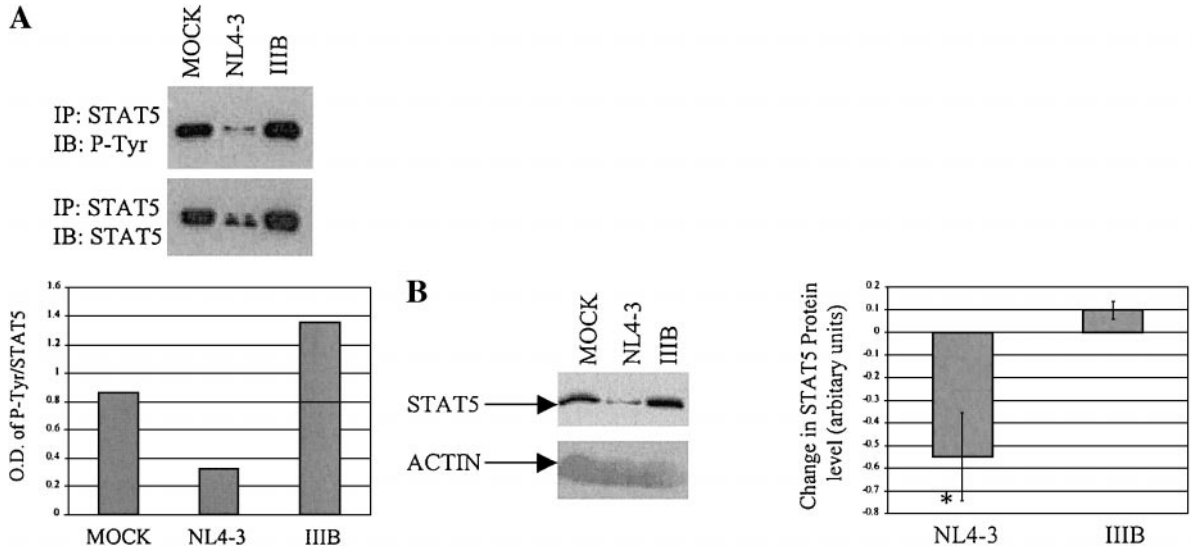

FIG. 5. NL4-3, but not IIIB, inhibits STAT5 phosphorylation and expression. (A) STAT5 phosphorylation. $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB as in Fig. 1A. Cell lysates were immunoprecipitated (IP) with anti-STAT5 antibody. Nitrocellulose membrane was immunoblotted (IB) with anti-phosphotyrosine antibody (P-Tyr, top) and then stripped and immunoblotted with anti-STAT5 antibody (bottom). The OD of each band was determined and the ratio of P-Tyr/STAT5 was plotted. $p24^{gag+}$ cells in NL4-3- and IIIB-infected cultures were 45 and 35%, respectively. A representative experiment of three performed is shown. (B) STAT5 expression. $CD4^+$ T cells were infected with NL4-3 or IIIB as in Fig. 1A. Cell lysates were analyzed by immunoblotting with anti-STAT5 antibody (top) and then the nitrocellulose membrane was stripped and immunoblotted with anti-actin mAb (bottom). The OD of each band was determined and change in STAT5 protein level in HIV-infected T cells was compared to the level in uninfected T cells, calculated as described under Materials and Methods. The blot shown is a representative experiment of four and the graph is average ± SEM of four experiments. The asterisk represents a significant difference between NL4-3 and IIIB ($P = 0.04$).

NL4-3 does not inhibit STAT1 phosphorylation or expression

Experiments were done to determine whether NL4-3 or IIIB infection of T cells inhibits tyrosine phosphorylation of a different STAT member, STAT1. PHA-activated $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB and STAT1 phosphorylation was determined as described under Materials and Methods. Briefly, postnuclear lysates were immunoprecipitated with anti-STAT1 mAb and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 7A, neither NL4-3 nor IIIB infection inhibited tyrosine phosphorylation of STAT1. Numbers of productively infected cells in NL4-3 and IIIB cultures were similar.

Similar experiments were done to determine the expression of STAT1 in NL4-3- or IIIB-infected T cells. STAT1 expression was determined by separating postnuclear lysates by SDS–PAGE and immunoblotting with anti-STAT1 mAb and then with anti-actin mAb. The optical density of each band was measured and the ratio of STAT1/actin was graphed. As shown in Fig. 7B, neither NL4-3 nor IIIB infection inhibited STAT1 expression. These data suggest that NL4-3 specifically targets the JAK3/STAT5 activation pathway.

DISCUSSION

Biochemical mechanisms of HIV-1-induced T cell dysfunction and apoptosis remain controversial. Here, we show that the JAK3/STAT5 activation pathway is inhibited in $CD4^+$ T cells infected with NL4-3, while productive infection with HIV-1 IIIB does not inhibit JAK3/STAT5 activation. Recent work from our laboratory has shown that $CD4^+$ T cells productively infected with HIV-1 strain IIIB have a lower number of apoptotic cells than $CD4^+$ T cells productively infected with NL4-3 (Rapaport *et al.,* 1998). Of note, the frequency of apoptotic IIIB-infected cells is even less than that seen in uninfected cultures (Rapaport *et al.,* 1998). Our earlier work analyzed apoptosis and infection in lymph nodes from HIV-infected individuals, a major reservoir of viral infection. Surprisingly, apoptosis was found predominantly in uninfected bystander cells and not in productively infected cells (Finkel *et al.,* 1995). While the work reported here did not analyze primary viral isolates, it is intriguing to speculate that these isolates behave like IIIB and inhibit apoptosis in infected cells. The biological significance of the difference between the two T-tropic viruses is not known. We hypothesize that a mutation(s) may have occurred in IIIB which protects the infected cells from apoptosis. Maintenance of host cell viability would provide an advantage to the virus by allowing production of more virions. The biochemical mechanisms involved in inhibition of apoptosis by HIV-1 IIIB are not understood. As shown in Fig. 3, the JAK3 protein level is higher in IIIB-infected T cells than in uninfected T cells. Although the significance of this higher JAK3 level is not known, we hypothesize that activation of the JAK3/STAT5 pathway protects IIIB in-

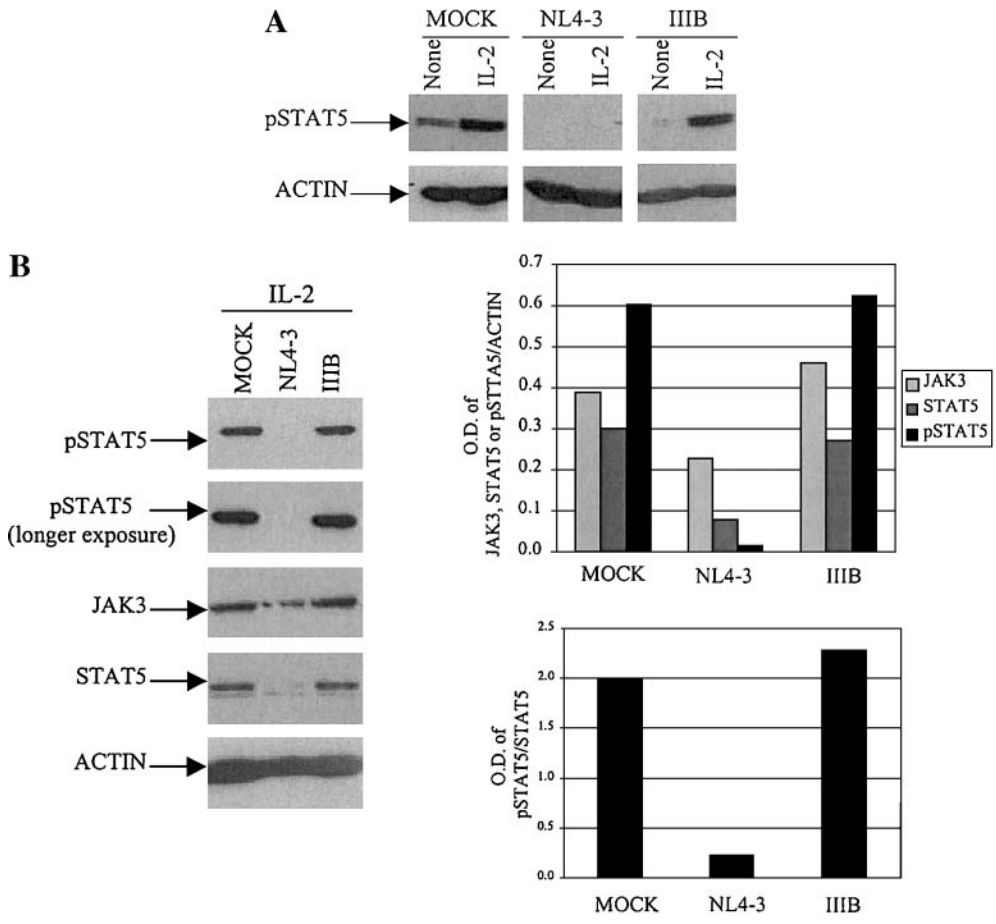

FIG. 6. NL4-3, but not IIIB, inhibits JAK3/STAT5 activation. (A) $CD4^+$ T cells were infected with NL4-3 or IIIB as in Fig. 1A. At day 6 postinfection, 5×10^5 cells were incubated without or with 50 U/ml IL-2 at 37°C for 15 min. Cells were lysed and postnuclear lysates were analyzed by immunoblotting with phosphorylation-specific anti-STAT5 mAb (pSTAT5, top). The nitrocellulose membrane was stripped and immunoblotted with an anti-actin antibody (bottom). A representative experiment of two performed is shown. (B) 5×10^5 cells were incubated with 50 U/ml IL-2 at 37°C for 15 min. Cells were lysed and postnuclear lysates were analyzed by immunoblotting with anti-pSTAT5 mAb. The nitrocellulose membrane was stripped and immunoblotted with anti-JAK3, anti-STAT5, and then anti-actin antibodies. The pSTAT5 blot was exposed to the film for longer times (pSTAT5—longer exposure). The OD of each band was determined and the ratios of JAK3, STAT5, pSTAT5/actin, or pSTAT5/STAT5 were plotted. A representative experiment of two performed is shown.

fected cells from apoptosis. Further work is needed to test this hypothesis.

NL4-3-infected T cells show inhibition of JAK3 and STAT5 activation and expression, which could lead to inhibition of IL-2-mediated proliferation and survival signals. As shown in Fig. 3, inhibition of JAK3 expression correlates with apoptosis. Moderate inhibition of JAK1 activation is seen in NL4-3- but not IIIB-infected T cells. It has been reported recently that JAK3 phosphorylates JAK1 (Zhou *et al.,* 2000). This could explain the inhibition of JAK1 tyrosine phosphorylation in NL4-3-infected T cells. Interestingly, in contrast to JAK3, JAK1 protein expression is not inhibited in NL4-3-infected cells. Activation and expression of another Janus family kinase, JAK2, are not inhibited in NL4-3-infected T cells, arguing against a global inhibition of the JAKs. We hypothesize that the defect in JAK3 activation leads to inhibition of proliferation and induction of apoptosis in NL4-3-infected T cells. The defect in JAK3 activation may also block JAK1-mediated signaling; thus, JAK3 alone, or JAK3 and JAK1, is inhibited in transducing signals for STAT5 phosphorylation and/or other survival pathways, e.g., the PI3K/Akt cascade (Zhou *et al.,* 2000). Collectively, these data suggest that inhibition of the JAK3/JAK1/STAT5 activation pathway may explain, at least in part, the apoptosis seen in NL4-3-infected T cells. The biochemical mechanism(s) of inhibition of JAK3 expression in NL4-3-infected T cells is not understood. We hypothesize that NL4-3 gene products and/or binding of free virions to uninfected T cells are responsible for the JAK3 inhibition. Nef has been shown to bind to and inhibit $p56^{lck}$ and ERK kinase activity (Greenway *et al.,* 1996) and could, thus, inhibit TCR/CD3-induced JAK3 expression. Experiments are under way to test various NL4-3 gene products to determine their roles in inhibition of JAK3 activation and expression. Surprisingly, even a low level of NL4-3 infection inhibited JAK3 expression in T cells from infected cultures (data not shown). In previous work, we have

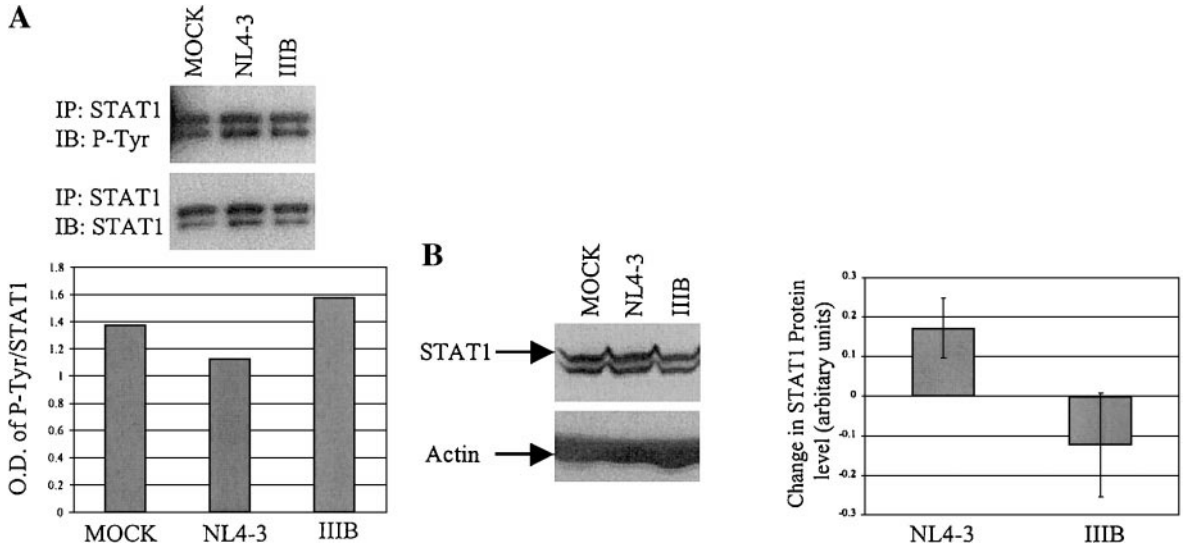

FIG. 7. NL4-3 and IIIB did not inhibit STAT1 phosphorylation or expression. (A) STAT1 activation. $CD4^+$ T cells were infected with HIV-1 NL4-3 or IIIB as in Fig. 1A. Cell lysates were immunoprecipitated (IP) with anti-STAT1 mAb. Nitrocellulose membrane was immunoblotted (IB) with anti-phosphotyrosine antibody (P-Tyr, top) and then stripped and immunoblotted with anti-STAT1 mAb (bottom). Percentages $p24^{gag+}$ cells in NL4-3- and IIIB-infected cultures were 26.29 and 23.73, respectively. The OD of each band was determined and the ratio of P-Tyr/STAT1 was plotted. A representative experiment of three performed is shown. (B) STAT1 expression. $CD4^+$ T cells were infected with NL4-3 or IIIB as in Fig. 1A. Cell lysates were analyzed by immunoblotting with anti-STAT1 mAb (top) and then the nitrocellulose membrane was stripped and immunoblotted with anti-actin mAb (bottom). Change in STAT1 protein level in HIV-infected T cells was compared to the level in uninfected T cells and calculated as described under Materials and Methods. The blot shown is a representative experiment of three and the graph is the average ± SEM of three experiments. There is no statistically significant difference between NL4-3 and IIIB ($P = 0.14$).

shown that increases in JAK3 expression and activation induced by antigen receptor ligation are inhibited by prior CD4 ligation by HIV gp120 or anti-CD4 mAb (Selliah and Finkel, 1998). It is possible that, in our cultures, viral particles or shed viral envelope protein ligated the CD4 receptor on uninfected cells, inducing a negative signal, and inhibition of JAK3 expression. Thus, productive infection of only a small number of T cells in HIV-infected individuals could affect JAK3/STAT5 signaling of uninfected cells *in vivo,* leading to T cell dysfunction, death, and failure of regeneration of the T cell repertoire.

A reduced expression of STAT1α, STAT5A, and STAT5B has been reported in T cells from HIV-seropositive individuals (Pericle *et al.,* 1998). In addition, *in vitro* infection with HIV-1 (strain BZ167) inhibits STAT5 expression, but not STAT1 expression (Pericle *et al.,* 1998), in agreement with our findings (Figs. 5 and 7). In contrast, Bovolenta *et al.* reported that HIV-1 did not inhibit STAT5 expression in T cells (Bovolenta *et al.,* 1999). Although the basis for this discrepancy is not known, experimental differences between these studies include (1) analysis of different viral isolates (which, as shown here, can give markedly different results) and (2) the use of resting PBMCs, a system in which productive infection is limited (Unutmaz *et al.,* 1999). Interestingly, we have previously reported that CD4 ligation of resting T cells by HIV gp120 inhibits TCR/CD3-induced activation and expression of JAK3 (Selliah and Finkel, 1998). Our unpublished data show that CD4 ligation, without productive infection, inhibits STAT5 activation, but does not inhibit STAT5 expression (data not shown).

Our data suggest that productive infection with certain strains of HIV-1 inhibits activation of the JAK3/STAT5 pathway and, thus, may inhibit resultant transcriptional activation required for cell cycle progression, cell proliferation, and survival. The biochemical mechanisms of HIV-induced inhibition of the JAK3/STAT5 activation pathway are under investigation. We have reported that deletion of *nef, vpr,* and *vpu* genes did not completely rescue NL4-3-infected cells from apoptosis (Rapaport *et al.,* 1998). We hypothesize that these gene products may not be the sole mediators of inhibition of the JAK3/STAT5 activation pathway. Recent data suggest that inhibition of the JAK3/STAT5 activation pathway leads to inhibition of survival signals, possibly by failure of induction of Bcl-2 and/or Bcl-$_{XL}$ (Nosaka *et al.,* 1999; Suzuki *et al.,* 2000). It has been reported that Bcl-2 rescues T lymphopoiesis in γc-deficient mice (Kondo *et al.,* 1997). Binding of JAK3 to γc is required for its activation and JAK3- and γc-deficient mice show similar defects in T cell development and activation (Suzuki *et al.,* 2000). STAT5 activation has been shown to upregulate Bcl-$_{XL}$ by binding to its promoter (Horita *et al.,* 2000). STAT5 activation is necessary for cell cycle progression and antiapoptotic effects (Zamorano *et al.,* 1998). Collectively, these data show a correlation between apoptosis and inhibition of the JAK3/STAT5 activation pathway and may, in part, explain the T cell dysfunction and apoptosis seen in HIV disease.

Further experiments are under way to dissect the mechanistic differences between NL4-3 and IIIB in regulation of the JAK3/STAT5 activation pathway and apoptosis.

MATERIALS AND METHODS

Isolation of $CD4^+$ T cells

Heparinized venous blood obtained from healthy adult human donors was separated on a Ficoll–Paque (Pharmacia Biotech, Piscataway, NJ) gradient to obtain lymphocytes. $CD4^+$ T cells were isolated by incubation with anti-CD8 mAb (OKT8; ATCC, Manassas, VA), followed by negative selection on goat anti-mouse IgG-coated Immulan beads (Biotecx Laboratories, Houston, TX). Isolated cells were 80–95% $CD4^+$ by flow cytometric analysis (data not shown).

HIV-1 infection

Purified $CD4^+$ T cells (2×10^6 cells/ml) were activated with PHA (1.5 μg/ml; Murex Biotech Ltd., Kent, England) for 3 days in RPMI 1640 culture media (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini BioProducts, Calabas, CA). Cells (4×10^6) were incubated with HIV molecular clone NL4-3 (obtained through NIH AIDS Research and Reference Reagent Program, Bethesda, MD) at multiplicity of infection (m.o.i.) of 0.05 or with HIV-1 strain IIIB (ABI, Colombia, MD) at m.o.i. of 0.05 for 2 h at 37°C. Cells were washed once and resuspended at 1×10^6 cells/ml in culture media with 20 U/ml interleukin-2 (R&D Systems, Minneapolis, MN). The cell concentration was adjusted to 1×10^6/ml every 2–3 days. Productive infection of T cells was monitored by staining for expression of intracytoplasmic protein, $p24^{gag}$, with phycoerythrin-conjugated anti-p24 mAb (KC57-RD1; Coulter, Hialeah, FL).

In some experiments, after 4 days of infection with NL4-3 or IIIB, T cells were activated with anti-CD3 mAb. Three hundred microliters of cells at 2×10^6 per milliliter were added to a 24-well plate coated with anti-CD3 mAb (ATCC). Cells were incubated for 24 h at 37°C, harvested, and lysed for Western analysis as described below.

Immunoprecipitation and Western blotting

HIV-infected cells (5×10^6 cells) were harvested after 3, 4, or 6 days of infection and lysed in Tris buffer containing 1% NP-40, phosphatase inhibitors, and protease inhibitors. Postnuclear lysates were used for immunoprecipitation with the following antibodies in a sequential manner: anti-Jak1 polyclonal antibody (pAb; Upstate Biotechnology, Lake Placid, NY), anti-Jak2 pAb (Upstate Biotechnology), anti-JAK3 pAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-STAT1 mAb (Santa Cruz Biotechnology), and anti-STAT5 pAb (recognizes both isoforms STAT5a and STAT5b; Santa Cruz Biotechnology). The antibody–protein complex was pelleted using Sepharose-conjugated protein A (Sigma, St. Louis, MO) and boiled in sample buffer (0.4% SDS, 3% glycerol, and 1% β-mercaptoethanol (Sigma)) and the proteins were separated by 7.5% SDS–PAGE. The proteins were transferred to nitrocellulose membrane and immunoblotted with anti-phosphotyrosine mAb (P-Tyr, Ab-2; Oncogene Science, Cambridge, MA). Positive protein bands were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and SuperSignal Substrate (SSS; Pierce, Rockford, IL). Membranes were stripped with 62.5 mM Tris–HCl, pH 6.7, containing 10 mM β-mercaptoethanol and 2% SDS, immunoblotted with corresponding antibodies, and developed with HRP-conjugated protein A and SSS.

In other experiments, 1×10^6 cell equivalents of postnuclear lysate were boiled with sample buffer and separated by 7.5% SDS–PAGE. Nitrocellulose membrane was immunoblotted with the following antibodies: anti-JAK1 pAb, anti-JAK2 pAb, anti-JAK3 pAb, anti-STAT1 mAb, anti-STAT5 pAb, phosphorylation-specific anti-STAT5 mAb (pSTAT5; Zymed, San Francisco, CA), or anti-actin mAb (Sigma). The membrane was stripped between each immunoblotting as described above. Optical density of positive bands was measured with the Geldoc 2000 (Bio-Rad, Hercules, CA).

Change in JAK3 protein level was calculated as follows:

$$\frac{\text{OD of JAK3}_{\text{HIV}} - \text{OD of JAK3}_{\text{MOCK}}}{\text{OD of JAK3}_{\text{MOCK}}}.$$

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